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Enzyme-Linked Immunosorbent Assays for Detection of Equine Antibodies Specific to *Sarcocystis neurona* Surface Antigens†

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Sarcocystis neurona **is the primary causative agent of equine protozoal myeloencephalitis (EPM), a common neurologic disease of horses in the Americas. We have developed a set of enzyme-linked immunosorbent assays (ELISAs) based on the four major surface antigens of *S. neurona* (SnSAGs) to analyze the equine antibody response to *S. neurona*. The SnSAG ELISAs were optimized and standardized with a sample set of 36 equine sera that had been characterized by Western blotting against total *S. neurona* parasite antigen, the current gold standard for *S. neurona* serology. The recombinant SnSAG2 (rSnSAG2) ELISA showed the highest sensitivity and specificity at 95.5% and 92.9%, respectively. In contrast, only 68.2% sensitivity and 71.4% specificity were achieved with the rSnSAG1 ELISA, indicating that this antigen may not be a reliable serological marker for analyzing antibodies against *S. neurona* in horses. Importantly, the ELISA antigens did not show cross-reactivity with antisera to *Sarcocystis fayeri* or *Neospora hughesi*, two other equine parasites. The accuracy and reliability exhibited by the SnSAG ELISAs suggest that these assays will be valuable tools for examining the equine immune response against *S. neurona* infection, which may help in understanding the pathobiology of this accidental parasite-host interaction. Moreover, with modification and further investigation, the SnSAG ELISAs have potential for use as immunodiagnostic tests to aid in the identification of horses affected by EPM.**

Sarcocystis neurona is a coccidian parasite that can infect horses and occasionally cause the neurologic disease equine protozoal myeloencephalitis (EPM) (6, 9). Horses become infected with *S. neurona* by ingesting sporocyst-contaminated food and water sources (8, 15). Ultimately, *S. neurona* can invade the central nervous system of the infected horse, causing focal or multifocal inflammation and EPM. *S. neurona* infection in horses is assessed by the detection of antibodies against the parasite in either the serum or cerebrospinal fluid (CSF); however, not all horses that seroconvert to *S. neurona* will develop EPM (9, 27). The seroprevalence of *S. neurona* infection in horses in the United States ranges between 0 and 89.2%, depending upon geographic locale (1–3, 10, 34, 37, 39, 40). In contrast, the incidence of clinical EPM has been estimated at <1% (28). It is not well understood what factors are responsible for the dichotomy between inapparent infection and clinical disease, but this ambiguity creates a major hindrance to EPM diagnosis and disease control.

Current technologies for detecting *S. neurona* antibodies in equine serum and CSF samples include Western blotting (17), a modified version of Western blotting (35), an *S. neurona* direct-agglutination test (SAT) (25), and an indirect fluorescent-antibody test (5). Each of these current serodiagnostic assays utilizes complete *S. neurona* merozoite preparations as the antigen source, which has several drawbacks. Specifically, propagation of parasite cultures is relatively time-consuming and expensive, and the use of whole-parasite preparations can increase the risk of false-positive results due to cross-reactivity with closely related pathogens, such as *Sarcocystis fayeri* (11, 38). Additionally, the current assays are not very amenable to quantitation, and their results can be subject to interpretation (16, 32). Given these shortcomings, a detailed and in-depth characterization of equine humoral responses to *S. neurona* infection is not feasible with the existing serologic tests.

Four related surface antigens have been identified in *S. neurona* merozoites, and these have been designated SnSAG1, SnSAG2, SnSAG3, and SnSAG4 (13, 20). To develop better tools for analyzing antibody responses to *S. neurona* infection, antibody capture enzyme-linked immunosorbent assays (ELISAs) were designed to utilize recombinant forms of the four *S. neurona* surface antigens (rSnSAGs). Comparison of the rSnSAG ELISAs with Western blot analysis of *S. neurona* merozoites confirmed that three of these assays are highly accurate and reliable. These ELISAs will serve as valuable tools for the evaluation of the equine humoral immune response to *S. neurona* infection, which may in turn allow discrimination between horses with EPM and those with asymptomatic *S. neurona* infections.

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MATERIALS AND METHODS

Parasite culture. The SN3 strain of *S. neurona* and the Oregon strain of *Neospora hughesi* (7, 18) were maintained by serial passage in bovine turbinate cell monolayers. Upon lysis of the host cell monolayer, zoites were passed twice

through 20-gauge (20-G), 22-G, and 25-G needles and filtered through a 3.0-μm Nucleopore (Whatman) membrane to remove host cell debris. The harvested parasites were counted with a hemocytometer, washed with phosphate-buffered saline (PBS), and stored at −20°C.

Recombinant-protein preparation. The four SnSAGs were expressed as recombinant proteins and purified by nickel column chromatography, as described previously (20). The concentration of the purified protein was determined by a colorimetric assay (Coomassie Plus Protein Assay Reagent; Pierce). Purified rSnSAG1, rSnSAG2, rSnSAG3, and rSnSAG4 were each diluted in elute buffer (0.5 M NaCl and 20 mM Tris-HCl) without urea to final protein concentrations of 8.15 μg/ml, 23.0 μg/ml, 14.56 μg/ml, and 10.3 μg/ml, respectively.

Serum and CSF samples. The positive control serum samples were from two clinically affected horses that had histologically confirmed EPM. The negative control sample for all assays was a preinfection serum sample from a weanling used in an *S. neurona* infection trial (14). Thirty-six equine sera submitted to Equine Biodiagnostics (EBI)/IDEXX for *S. neurona* serology testing were used for standardization of the rSnSAG ELISAs. These samples had been classified as positive or negative in *S. neurona* Western blots using criteria established by EBI/IDEXX (9, 10). Samples from 27 EPM-confirmed horses were from a collection compiled at the University of Kentucky Gluck Equine Research Center. All cases were confirmed by histological examination of central nervous system tissues for the presence of lesions consistent with EPM, and prior Western blot analyses had demonstrated that all 27 horses had CSF antibodies against *S. neurona*. Paired serum and CSF samples were available for most EPM horses, with the exception of numbers 2, 5, 6, 14, and 16, for which CSF samples were absent, and horse 27, for which serum was absent. Three equine serum samples from an *S. fayeri* challenge trial (38) were used to examine assay cross-reactivity. An *N. hughesi* positive control serum sample was taken from a horse that was naturally infected with *N. hughesi* (30) and was provided by A. E. Marsh, Ohio State University.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Native or recombinant proteins were suspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer supplemented with protease inhibitor cocktail (Sigma) and separated on 12% polyacrylamide gels (24). For Western blot analysis, proteins were transferred to nitrocellulose membranes by semidry electrophoresis in Tris-glycine buffer, pH 8.3. Membranes were blocked with PBS containing nonfat dry milk, 0.1% Tween 20, and 5% normal goat serum (NGS), after which the membranes were incubated for 1 hour in primary antibody solution. The membranes were washed, followed by incubation for 45 min with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Membranes were processed for chemiluminescent detection using SuperSignal substrate (Pierce) and exposed to radiographic film or documented with a FluorChem 8800 imaging system (Alpha Innotech, Corp.).

ELISA. The rNhSAG1 ELISA was performed as described previously (19). For rSnSAG ELISAs, high-binding 96-well plates (Corning) were incubated overnight at 4°C with 100 μl purified rSnSAG1, rSnSAG2, rSnSAG3, or rSnSAG4 diluted to 0.20 μg/ml, 1.00 μg/ml, 0.09 μg/ml, and 0.21 μg/ml, respectively. The plates were rinsed three times with PBS containing 0.05% Tween 20 (PBST) and blocked for 1.5 h at room temperature (RT) with PBS containing 1% Tween 20, 5% NGS, and 0.01 g/ml nonfat dry milk. Primary sera or CSF was diluted to the appropriate concentration in PBS containing 0.1% Tween 20, 0.5% NGS, and 0.001 g/ml nonfat dry milk. One hundred-microliter aliquots of the antibody mixtures were added to duplicate wells of the plate and incubated for 2 hours at RT. The wells were rinsed five times with PBST and incubated for 2 hours at RT with 150 μl of horseradish peroxidase-conjugated goat anti-horse immunoglobulin G (IgG) secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) diluted to 1:10,000 in PBS containing 0.1% Tween 20, 0.5% NGS, and 0.001 g/ml nonfat dry milk. The wells were then rinsed four times with PBST. The chromogenic substrate *o*-phenylenediamine dihydrochloride (Sigma) was dissolved in 0.5 M phosphate-citrate buffer to a concentration of 0.4 mg/ml, and 200 μl of substrate solution was added to each well. After a 10-minute incubation, the reaction was stopped with 50 μl of 3 M H_2SO_4, and the optical density at 490 nm (OD_{490}) was measured in an E_{max} microplate reader (Molecular Devices). To account for interplate variation, the OD of each serum sample was expressed as a percentage of the high positive standard on the plate, calculated as described in the following equation (41): PP(%) = [OD(sample) − OD(NC)]/[OD(PC) − OD(NC)] × 100, where PP is the percent positivity of each sample, NC is the average negative control OD, and PC is the average positive control OD.

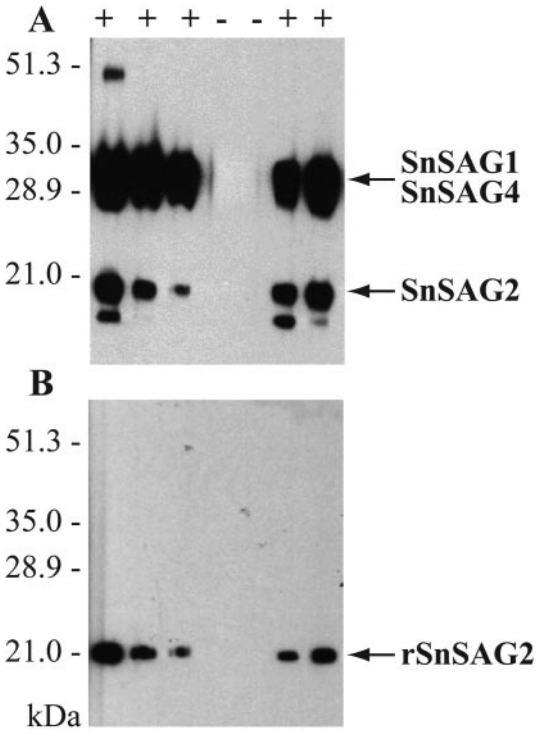

FIG. 1. Parallel Western blot analyses of (A) whole reduced *S. neurona* lysate (5×10^6 merozoites) and (B) 0.8 μg rSnSAG2 protein showed good correlation between CSF antibody recognition of the rSnSAG2 and native parasite antigen. The immunodominant antigens representing SnSAG1, SnSAG4, and SnSAG2 are indicated, as described previously (20). With the Western blot conditions used, native SnSAG3 was not visible. CSF samples were run at 1:25. +, confirmed EPM positive; −, confirmed EPM negative.

RESULTS

Recombinant-protein antibody recognition by Western blotting. Equine antibody reactivity with the four rSnSAGs was initially analyzed in Western blots and compared to Western blot results for *S. neurona* whole-parasite antigen. As shown in Fig. 1, CSF from five seropositive horses showed antibody recognition of rSnSAG2, whereas two seronegative horses did not react to the recombinant antigen. Importantly, there was no reactivity to other proteins that might be minor contaminants of the purified antigen preparation. Similar results were obtained with rSnSAG1, rSnSAG3, and rSnSAG4 (data not shown).

Standardization of rSnSAG ELISAs. Initially, checkerboard titrations were used to determine the optimal recombinant-protein concentrations and secondary-antibody dilutions for each rSnSAG ELISA. Under these defined conditions, significant background occurred with primary serum dilutions of less than 1:250, so this was selected as the starting dilution for the four assays. To standardize the ELISAs, total serum IgG antibody reactivity to each of the four surface antigens was determined on a sample set of 36 horse sera that had been previously characterized by Western blotting. Samples were tested with the ELISAs at a 1:250 dilution, and the mean OD_{490} value for each sample was converted into a PP value. To determine the optimal cutoff for each ELISA, sensitivity and

TABLE 1. Accuracies of the rSnSAG ELISAs at different PP cutoff values

PP	Sensitivity/specificity (%)[a]			
	rSnSAG1	rSnSAG2	rSnSAG3	rSnSAG4
5	68.2/50	100/14.3	100/14.3	100/14.3
10	68.2/71.4	100/57.1	90.9/42.6	100/50
15	54.5/85.7	95.5/78.6	90.9/78.6	95.5/85.7
20	45.5/92.9	95.5/92.9	77.3/85.7	90.9/85.7
25	45.5/100	90.9/100	72.7/100	90.9/85.7
30	45.5/100	81.8/100	63.6/100	86.4/100
35	40.9/100	72.7/100	54.5/100	81.8/100

[a] Calculations based on comparison to Western blot results for the 36 serum samples used for standardization of the ELISAs.

specificity were calculated at different PP values by comparison to Western blot results for the 36 samples (Table 1). Based on these evaluations, the rSnSAG2 ELISA showed the highest accuracy of 95.5% sensitivity and 92.9% specificity when a PP cutoff of 20% was used. In contrast, only 68.2% sensitivity and 71.4% specificity were achieved with the rSnSAG1 ELISA at its optimum PP cutoff of 10%. A PP of 15% was selected as the optimal cutoff for both the rSnSAG3 and the rSnSAG4 ELISAs (Table 1).

Analysis of EPM-confirmed serum samples. To investigate the total IgG serum antibody response in clinically affected horses, 26 samples from EPM-confirmed horses were tested in twofold dilutions from 1:250 to 1:8,000, and the previously defined optimal cutoff value for each rSnSAG ELISA was utilized to determine the end point titer for each sample. Serum antibody titers against rSnSAG4 were detected in 25 of the 26 (96.2%) EPM horses (Fig. 2). The rSnSAG2 and rSnSAG3 ELISAs yielded seropositive results in 24 of 26 (92.3%) serum samples. Only 18 of the 26 (69.2%) EPM horses had detectable serum antibody titers against rSnSAG1. Interestingly, some serum samples exhibited considerable variation in the antibody titer detected against each of the rSnSAGs (Fig. 2), suggesting that individual animals may generate unequal responses to the different *S. neurona* surface antigens.

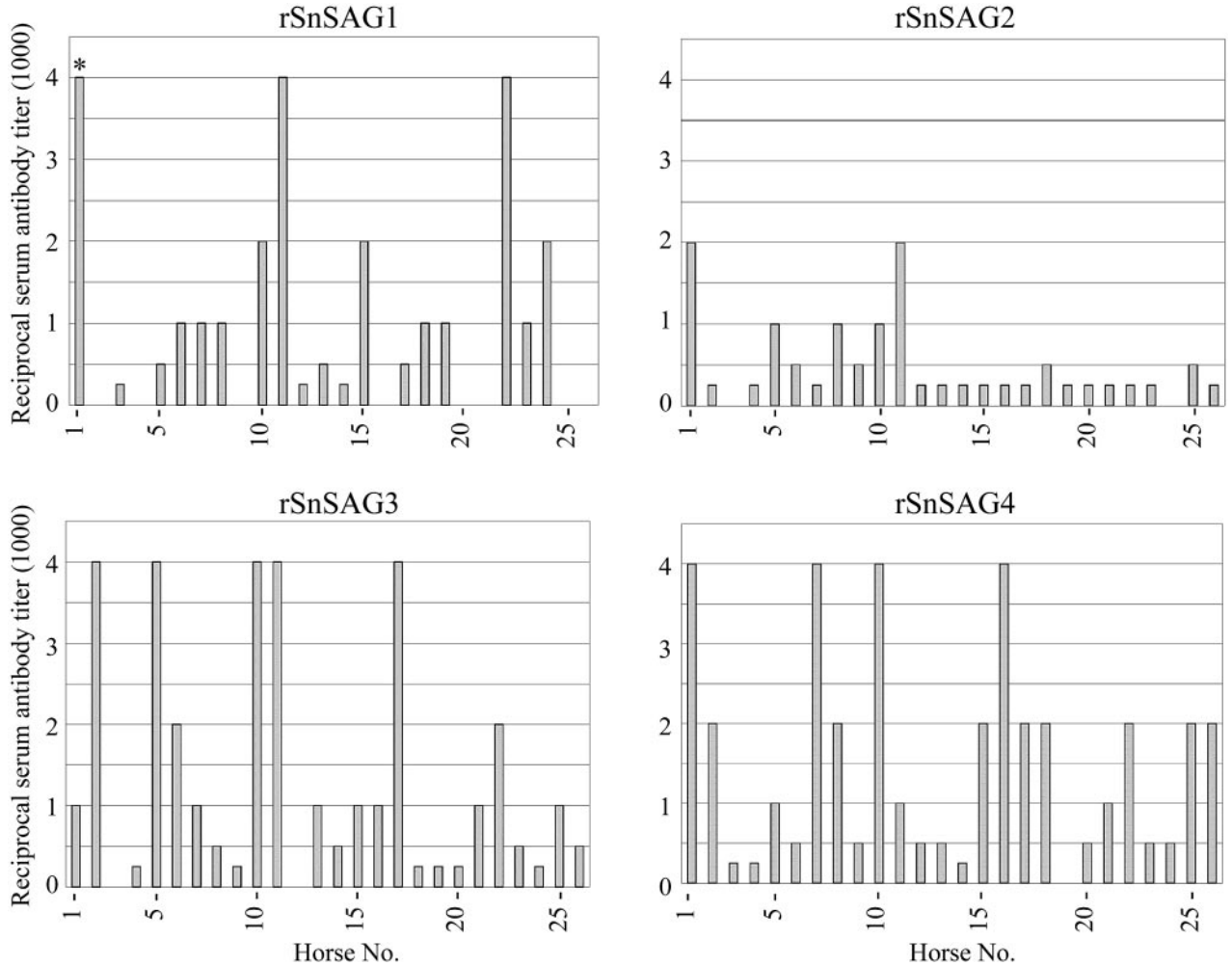

FIG. 2. Reciprocal antibody titers determined by ELISA analysis of serum samples from 26 EPM-confirmed horses revealed that 24, 24, and 25 sera had detectable antibody titers to rSnSAG2, rSnSAG3, and rSnSAG4, respectively. An antibody titer to rSnSAG1 was detected in only 18 of the 26 sera. All samples were tested in duplicate in serial twofold dilutions ranging from 1:250 to 1:8,000. The end point titer was the final dilution at which the PP was greater than the optimal cutoff defined for each SnSAG ELISA. *, titer of 1:8,000.

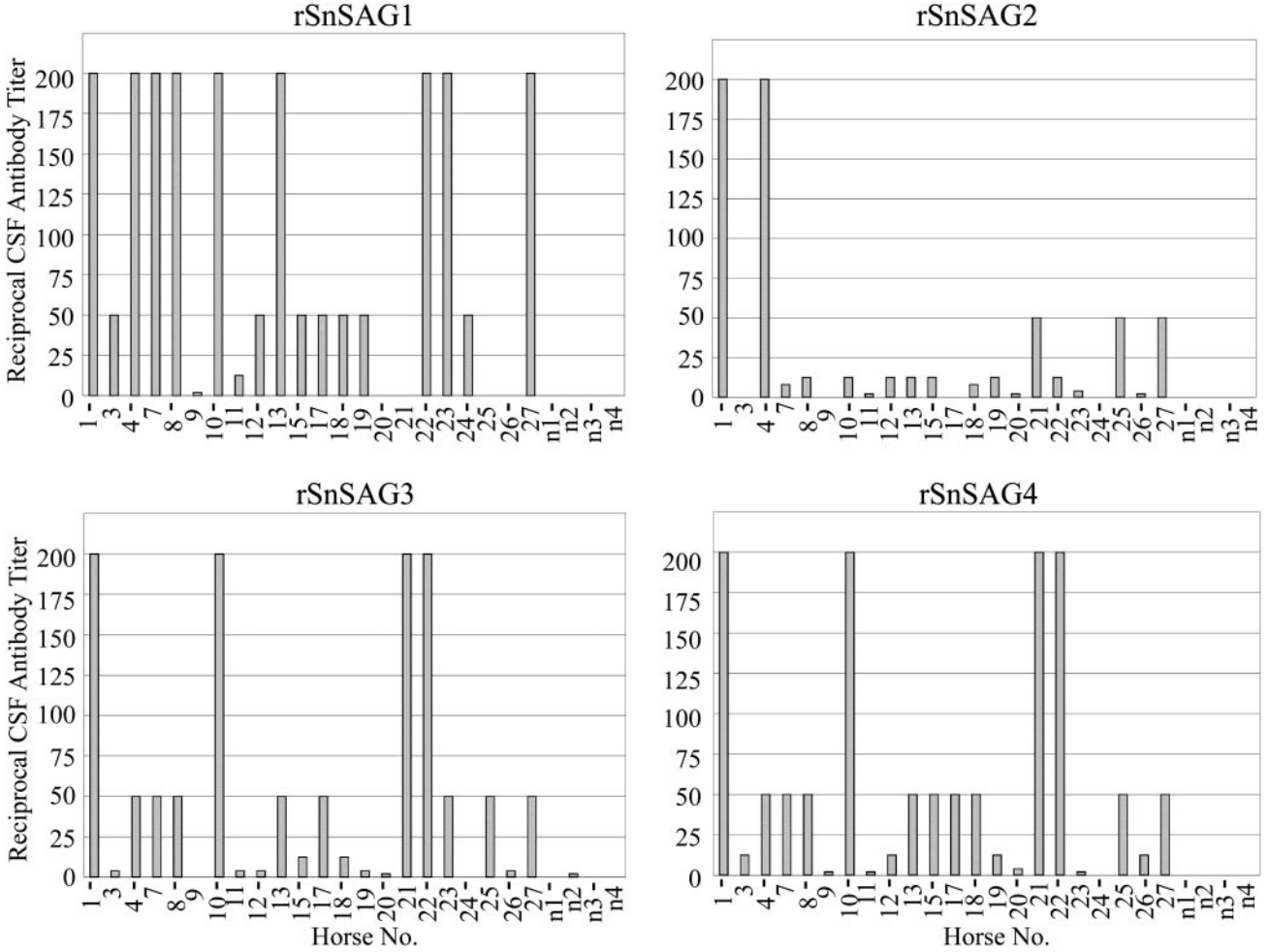

FIG. 3. Reciprocal antibody titers determined by ELISA analysis of CSF from 22 EPM-confirmed horses and four seronegative horses demonstrated that 18, 18, 20, and 21 of the 22 samples had detectable CSF antibodies against rSnSAG1, rSnSAG2, rSnSAG3, and rSnSAG4, respectively. Samples were tested in duplicate in serial dilutions ranging from 1:2 to 1:200. The reciprocal CSF antibody titer was determined by the lowest dilution at which the PP was greater than or equal to the defined cutoff for each rSnSAG ELISA. CSF sample numbering is the same as for the serum samples in Fig. 4 with the addition of horse 27, for which no paired serum was available. Samples n1, n2, n3, and n4 are from the four seronegative horses.

Analysis of EPM-confirmed CSF samples. To evaluate the total IgG antibody response in the CSF of clinically affected horses, CSF samples from 22 of the EPM-confirmed horses and four seronegative horses were tested at multiple dilutions against the four surface antigens. Due to limited CSF quantities, the samples were initially tested at dilutions of 1:12.5, 1:50, and 1:200. Samples that did not have a detectable antibody titer at the 1:12.5 dilution were subsequently retested at 1:2, 1:4, and 1:8 dilutions. Any sample at the 1:2 dilution with a PP greater than or equal to the defined cutoff for each ELISA was considered antibody positive. In total, 18 (81.8%), 18 (81.8%), 20 (90.9%), and 21 (95.5%) of the 22 CSF samples had detectable antibody titers against rSnSAG1, rSnSAG2, rSnSAG3, and rSnSAG4, respectively (Fig. 3). One negative control sample (horse n2) exhibited antibody reactivity to rSnSAG3 at the 1:2 dilution, but this sample was negative to rSnSAG1, rSnSAG2, and rSnSAG4. With the exception of five horses, very low CSF antibody titers against rSnSAG2 were observed. In contrast, CSF antibody titers were generally higher against rSnSAG1, rSnSAG3, and rSnSAG4. Similar to the serum results, considerable variation in the antibody titer to each of the four SnSAGs was observed in some samples (Fig. 3).

Assessment of antibody cross-reactivity. To determine whether the rSnSAGs cross-react with antibodies against closely related apicomplexan parasites, serum samples from three horses challenged with *S. fayeri* were tested in the ELISAs (38). A total of 13 serum samples were taken weekly from each horse during the duration of the trial. Although interpretation for *S. neurona*-specific serology was not hindered (38), Western blot analysis clearly demonstrated that *S. fayeri*-infected horses produce antibodies that cross-react with multiple *S. neurona* antigens (Fig. 4A). When tested with the ELISAs at a 1:250 dilution, the serum samples exhibited no consistent or appreciable rise in antibody reactivity to the rSnSAGs over the course of the experiment (Fig. 4B). Although the calculated PP values occasionally equaled or eclipsed the cutoffs at one or a few time points, the antibody

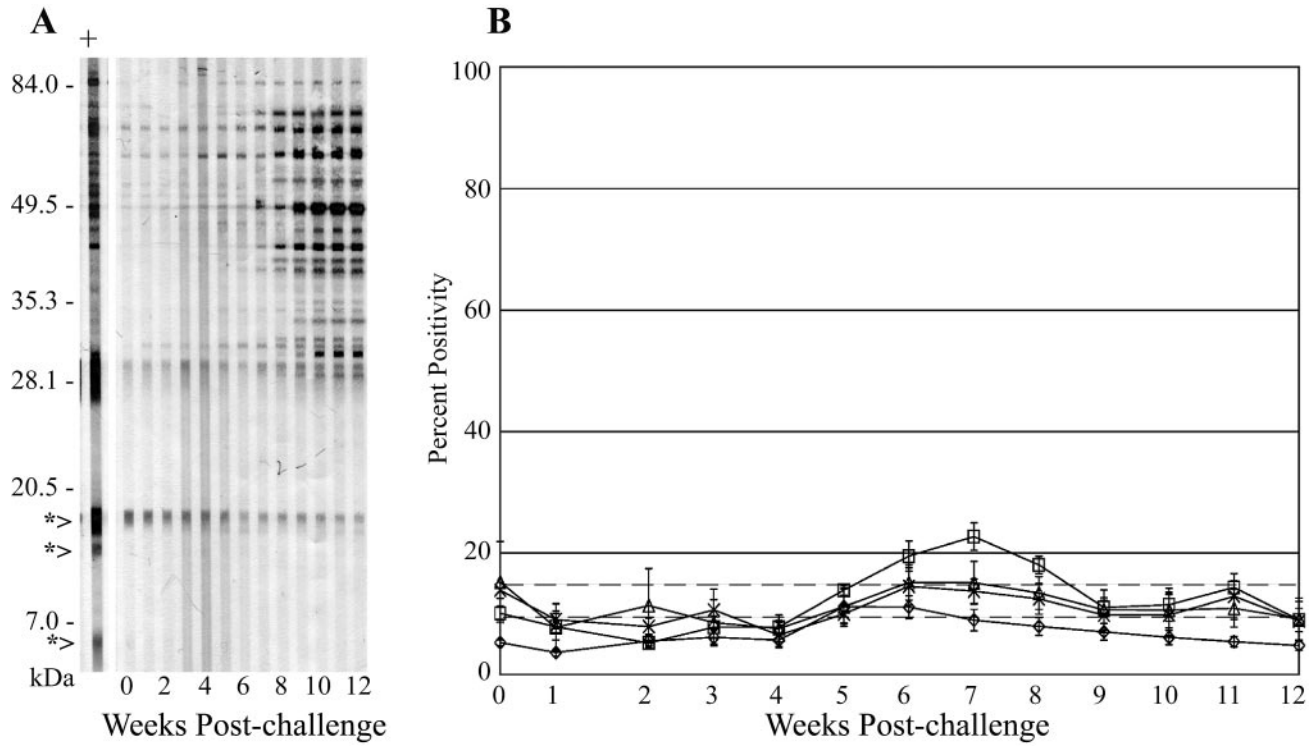

FIG. 4. Serum antibody analysis of horses challenged with *S. fayeri* (38) demonstrated that anti-*S. fayeri* sera at 1:250 dilution do not react with the four rSnSAGs. (A) A representative Western blot of *S. neurona* merozoites probed with serum samples collected over time from an *S. fayeri*-challenged horse showed increasing cross-reactivity with numerous undefined parasite antigens. +, positive control serum; * indicates the antigens used as the basis for *S. neurona* serology (EBI/IDEXX). (B) Mean ELISA PP values (± standard errors of the mean) for the three *S. fayeri* challenge horses revealed no consistent rise in antibody reactivity over the course of 13 weeks, thus indicating that anti-*S. fayeri* sera do not cross-react with the four rSnSAGs. The dotted lines indicate PPs of 10 and 15. ◇, rSnSAG1; □, rSnSAG2; △, rSnSAG3; ×, rSnSAG4.

reactivity was generally below the defined cutoffs for each of the assays.

Two of the 36 validation samples, numbers 27 and 33, were seropositive to *N. hughesi* by both ELISA and Western blotting (19). However, neither of these horses had a significant serologic response to the rSnSAGs (Fig. 1). Additionally, the serum and CSF from a horse that had been naturally infected with *N. hughesi* (29) were tested in the rSnSAG ELISAs. Previously, this horse had exhibited slight seroreactivity to *S. neurona* by Western blotting, suggesting prior exposure to the parasite (29). The ELISA analyses demonstrated that this horse had a serum titer of 1:500 to rSnSAG2 and 1:250 to rSnSAG4 (data not shown), which are consistent with the Western blot results. However, the horse was seronegative to the other two rSnSAGs and showed no CSF antibody reactivity to any of the rSnSAGs (Fig. 3, horse n4). Collectively, these results indicated that antibodies against two related equine pathogens, *S. fayeri* and *N. hughesi*, will not confound the rSnSAG ELISA results.

DISCUSSION

There is substantial precedent for utilizing the surface antigens of apicomplexan parasites in sensitive serodiagnostic tests (4, 19, 21, 23, 36). In this study, we developed ELISAs based on the immunodominant surface antigens of *S. neurona* (20), and these assays have proven accurate for detecting antibodies to *S. neurona* in equine sera and CSF. Importantly, the assays do not cross-react with antisera against *S. fayeri*, a closely related species that utilizes the horse as a natural intermediate host (11). The ELISA offers many advantages over current *S. neurona* serologic assays, including ease of use, high sample throughput, and more objective interpretation of results. Furthermore, the use of recombinant antigens in the ELISAs precludes the need for propagation of parasites in tissue cultures. The rSnSAG2, rSnSAG3, and rSnSAG4 ELISAs, in particular, will be important tools for the in-depth examination of the equine humoral response to *S. neurona* infection. With some modifications, such as combining SnSAGs in a single ELISA, and with further investigation using larger serum sets with more negative samples, these assays may also prove useful as serodiagnostic tests, as they showed high sensitivity and specificity compared to both Western blot serodiagnosis and postmortem diagnosis.

A variety of serologic assays have been developed to test for the presence of anti-*S. neurona* antibodies in serum or CSF. These include an SAT (25), which assesses antibody agglutination of formalin-fixed *S. neurona* merozoites, and an indirect fluorescent-antibody test (5), which monitors the presence of antibodies by fluorescence of merozoites. Both of these assays are well designed for testing large numbers of samples and determining end point titrations. The SAT has the added benefit of being useful for testing sera from other animal species

without assay modification. Western blot analysis of total merozoite protein, also referred to as the immunoblot assay, was the first assay used for detecting serum and CSF antibodies against *S. neurona*. Various permutations of the Western blot assay have been reported and utilized (35), but the immunoblot assay as originally described remains the most commonly used test for EPM immunodiagnosis (17). This assay was initially described in the early 1990s, and is based on antibody recognition of low-molecular-weight antigens of unknown identity (9, 10). The immunoblot assay provides a wealth of information, thereby providing enhanced confidence in the accuracy of the assay results. It is for this reason that Western blotting is often used as the gold standard for serologic testing.

Unfortunately, there is an assortment of shared or unique limitations that are associated with each of the serologic assays described above. A number of these drawbacks are partially or totally alleviated in the rSnSAG ELISAs. The above-mentioned need for culture-derived *S. neurona* merozoites is negated by using the rSnSAGs as the antigen source in these ELISAs. Relative to propagation of *S. neurona* in tissue culture, production of these recombinant proteins is very simple and inexpensive. For example, a 500-ml culture of bacteria yielded 30 mg of rSnSAG2 protein, which is sufficient to test greater than 100,000 serum or CSF samples in duplicate. Additionally, the use of a specific defined antigen or epitope that is known to be immunodominant reduces the likelihood of cross-reactivity in serologic tests for other apicomplexan parasites (21, 23, 26, 33). Despite obvious antigenic cross-reactivity between *S. neurona* and *S. fayeri*, the rSnSAG ELISAs were not confounded by the presence of anti-*S. fayeri* antibodies (Fig. 4). Most significantly, the ELISA format utilizes an objective cutoff and is very amenable to modification for analysis of specific antibody subsets (i.e., isotypes). Consequently, the rSnSAG ELISAs will allow a more in-depth characterization of the equine immune response to *S. neurona* infection.

One noteworthy and surprising result of these studies is that serum antibody titers to rSnSAG1 could be detected in only 68.2% of the positive validation horses and 69.2% of the EPM horses. Consistent with these results, some serum samples tested by Western blotting at EBI/IDEXX fail to demonstrate reactivity with the immunodominant 30-kDa antigen that is primarily SnSAG1 (13, 20) despite reacting to multiple other antigens, including those that are diagnostic (J. K. Morrow, unpublished data). The prototypic SnSAG1 has been found to be absent in cultured isolates of *S. neurona* from two EPM horses (22, 31; D. K. Howe, *S. neurona* EST sequencing project), and it is conceivable that this surface antigen polymorphism occurs in a proportion of *S. neurona* strains in nature. Although a serodiagnostic assay has been developed based on a recombinant form of SnSAG1 (12), our study indicates that this antigen may not be the most reliable serological marker for detecting *S. neurona* infection in horses. Currently, there is no evidence of strain variation at the other three surface antigen loci of *S. neurona*. As such, SnSAG2, SnSAG3, and SnSAG4 should serve as dependable markers for serologic testing.

Elucidation of the factors that influence whether an *S. neurona* infection will result in neurologic disease would greatly enhance our understanding of EPM pathogenesis and the identification of EPM-afflicted horses. The described ELISAs will be useful for examining the equine humoral immune response against specific parasite surface antigens during *S. neurona* infection. With the rSnSAG ELISAs, changes in antibody titers can be easily followed throughout the course of infection and disease progression. Also, studies using these assays are under way to analyze specific antibody isotype responses against the SnSAGs, thereby determining whether disparity in humoral immune responses exists between horses that are seropositive but clinically unaffected and horses that are afflicted with EPM.

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